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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number
WO 02/22632 A2

(51) International Patent Classification[7]: C07H 21/00

(21) International Application Number: PCT/EP01/10210

(22) International Filing Date:
5 September 2001 (05.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
100 44 948.4 12 September 2000 (12.09.2000) DE
101 20 094.3 25 April 2001 (25.04.2001) DE

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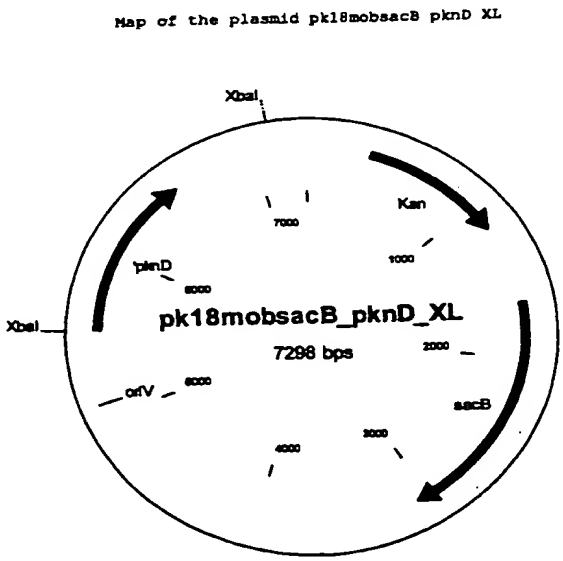
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NUCLEOTIDE SEQUENCES CODING FOR THE PKND GENE



(57) Abstract: The invention relates to an isolated polynucleotide which contains a polynucleotide sequence selected from the group comprising: a) a polynucleotide which is at least 70% identical to a polynucleotide coding for a polypeptide containing the amino acid sequence of SEQ ID No. 2, b) a polynucleotide coding for a polypeptide containing an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2, c) a polynucleotide which is complementary to the polynucleotides of a) or b), and d) a polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c), and a fermentation process for the preparation of L-amino acids using corynebacteria in which at least the pknD gene is amplified, and to the use, as hybridization probes, of polynucleotides containing the sequences according to the invention.

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Nucleotide Sequences Coding for the pknD Gene

Field of the Invention

The invention provides nucleotide sequences from corynebacteria coding for the pknD gene and a fermentation process for the preparation of amino acids using bacteria in which the endogenous pknD gene is amplified.

Prior Art

L-Amino acids, especially L-lysine, are used in human medicine, in the pharmaceutical industry, in the food industry and very especially in animal nutrition.

It is known that amino acids are prepared by the fermentation of strains of corynebacteria, especially Corynebacterium glutamicum. Because of their great importance, attempts are constantly being made to improve the preparative processes. Improvements to the processes may relate to measures involving the fermentation technology, e.g. stirring and oxygen supply, or the composition of the nutrient media, e.g. the sugar concentration during fermentation, or the work-up to the product form, e.g. by ion exchange chromatography, or the intrinsic productivity characteristics of the microorganism itself.

The productivity characteristics of these microorganisms are improved by using methods of mutagenesis, selection and mutant choice to give strains which are resistant to antimetabolites or auxotrophic for metabolites important in regulation, and produce amino acids.

Methods of recombinant DNA technology have also been used for some years to improve L-amino acid-producing strains of Corynebacterium by amplifying individual amino acid biosynthesis genes and studying the effect on amino acid production.

Object of the Invention

The object which the inventors set themselves was to provide novel measures for improving the preparation of amino acids by fermentation.

Summary of the Invention

When L-amino acids or amino acids are mentioned hereafter, they are understood as meaning one or more amino acids, including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Lysine is particularly preferred.

When L-lysine or lysine is mentioned hereafter, it is understood as meaning not only the bases but also the salts, e.g. lysine monohydrochloride or lysine sulfate.

The invention provides an isolated polynucleotide from corynebacteria which contains a polynucleotide sequence coding for the pknD gene and is selected from the group comprising:

a) a polynucleotide which is at least 70% identical to a polynucleotide coding for a polypeptide containing the amino acid sequence of SEQ ID No. 2,

b) a polynucleotide coding for a polypeptide containing an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2,

c) a polynucleotide which is complementary to the polynucleotides of a) or b), and

d) a polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of protein kinase D.

The invention also provides the above-mentioned polynucleotide, which is preferably a replicable DNA containing:

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence corresponding to sequence (i) within the degeneracy of the genetic code, or

(iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

(iv) neutral sense mutations in (i).

The invention also provides:

a replicable polynucleotide, especially DNA, containing the nucleotide sequence as shown in SEQ ID No. 1,

a polynucleotide coding for a polypeptide containing the amino acid sequence as shown in SEQ ID No. 2,

a vector containing the polynucleotide according to the invention, especially a shuttle vector or plasmid vector, and

corynebacteria which contain the vector or in which the endogenous pknD gene is amplified.

The invention also provides polynucleotides consisting substantially of a polynucleotide sequence which are obtainable by screening, by means of hybridization, of an appropriate gene library of a Corynebacterium, containing the complete gene or parts thereof, with a probe containing

the sequence of the polynucleotide of the invention according to SEQ ID No. 1 or a fragment thereof, and by isolation of said polynucleotide sequence.

Detailed Description of the Invention

As hybridization probes for RNA, cDNA and DNA, polynucleotides containing the sequences according to the invention are suitable for isolating the full length of nucleic acids, or polynucleotides or genes, coding for protein kinase D, or for isolating nucleic acids, or polynucleotides or genes, whose sequence exhibits a high degree of similarity to the sequence of the pknD gene. They are also suitable for incorporation into so-called arrays, micro-arrays or DNA chips for detecting and determining the corresponding polynucleotides.

Polynucleotides containing the sequences according to the invention are further suitable as primers for the preparation, by the polymerase chain reaction (PCR), of DNA of genes coding for protein kinase D.

Such oligonucleotides serving as probes or primers contain at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24 and very particularly preferably at least 15, 16, 17, 18 or 19 consecutive nucleotides. Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 or at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides may also be suitable.

"Isolated" means separated from its natural environment.

"Polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, it being possible for the RNAs or DNAs in question to be unmodified or modified.

the sequence of the polynucleotide of the invention according to SEQ ID No. 1 or a fragment thereof, and by isolation of said polynucleotide sequence.

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As hybridization probes for RNA, cDNA and DNA, polynucleotides containing the sequences according to the invention are suitable for isolating the full length of nucleic acids, or polynucleotides or genes, coding for protein kinase D, or for isolating nucleic acids, or polynucleotides or genes, whose sequence exhibits a high degree of similarity to the sequence of the pknD gene. They are also suitable for incorporation into so-called arrays, micro-arrays or DNA chips for detecting and determining the corresponding polynucleotides.

Polynucleotides containing the sequences according to the invention are further suitable as primers for the preparation, by the polymerase chain reaction (PCR), of DNA of genes coding for protein kinase D.

Such oligonucleotides serving as probes or primers contain at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24 and very particularly preferably at least 15, 16, 17, 18 or 19 consecutive nucleotides. Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 or at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides may also be suitable.

"Isolated" means separated from its natural environment.

"Polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, it being possible for the RNAs or DNAs in question to be unmodified or modified.

The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom, as well as polynucleotides which are in particular at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90% and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

"Polypeptides" are understood as meaning peptides or proteins containing two or more amino acids bonded via peptide links.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, especially those with the biological activity of protein kinase D and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90% and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polypeptide according to SEQ ID No. 2, and have said activity.

The invention further relates to a fermentation process for the preparation of amino acids selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine, using corynebacteria which, in particular, already produce amino acids and in which the nucleotide sequences coding for the pknD gene are amplified and, in particular, overexpressed.

In this context the term "amplification" describes the increase in the intracellular activity, in a microorganism, of one or more enzymes which are coded for by the appropriate DNA, for example by increasing the copy number of the gene(s) or allele(s), using a strong promoter or

using a gene or allele coding for an appropriate enzyme with a high activity, and optionally combining these measures.

By amplification measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

The microorganisms provided by the present invention can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch or cellulose or from glycerol and ethanol. Said microorganisms can be representatives of corynebacteria, especially of the genus Corynebacterium. The species Corynebacterium glutamicum may be mentioned in particular in the genus Corynebacterium, being known to those skilled in the art for its ability to produce L-amino acids.

The following known wild-type strains:

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869, and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared therefrom, are particularly suitable strains of the genus Corynebacterium, especially of the species Corynebacterium glutamicum (C. glutamicum).

The novel pknD gene of C. glutamicum coding for the enzyme protein kinase D (EC 2.7.1.37) has been isolated.

The first step in isolating the pknD gene or other genes of C. glutamicum is to construct a gene library of this microorganism in Escherichia coli (E. coli). The construction of gene libraries is documented in generally well-known textbooks and manuals. Examples which may be mentioned are the textbook by Winnacker entitled From Genes to Clones, Introduction to Gene Technology (Verlag Chemie, Weinheim, Germany, 1990) or the manual by Sambrook et al. entitled Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A very well-known gene library is that of the E. coli K-12 strain W3110, which was constructed by Kohara et al. (Cell 50, 495-508 (1987)) in λ vectors. Bathe et al. (Molecular and General Genetics 252, 255-265, 1996) describe a gene library of C. glutamicum ATCC13032, which was constructed using cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA 84, 2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16, 1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326 (1992)) in turn describe a gene library of C. glutamicum ATCC13032 using cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)).

A gene library of C. glutamicum in E. coli can also be constructed using plasmids like pBR322 (Bolivar, Life Sciences 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene 19, 259-268). Restriction- and recombination-defective E. coli strains are particularly suitable as hosts, an example being the strain DH5αmcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can then in turn be subcloned into common vectors suitable for sequencing,

and subsequently sequenced, e.g. as described by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America 74, 5463-5467, 1977).

The DNA sequences obtained can then be examined with known algorithms or sequence analysis programs, e.g. that of Staden (Nucleic Acids Research 14, 217-232 (1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The novel DNA sequence of C. glutamicum coding for the pknD gene was found and, as SEQ ID No. 1, forms part of the present invention. Furthermore, the amino acid sequence of the corresponding protein was derived from said DNA sequence by the methods described above. The resulting amino acid sequence of the pknD gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 due to the degeneracy of the genetic code also form part of the invention. Likewise, DNA sequences which hybridize with SEQ ID No. 1 or portions of SEQ ID No. 1 form part of the invention. Furthermore, conservative amino acid exchanges, e.g. the exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are known to those skilled in the art as "sense mutations", which do not cause a fundamental change in the activity of the protein, i.e. they are neutral. It is also known that changes at the N- and/or C-terminus of a protein do not substantially impair its function or may even stabilize it. Those skilled in the art will find information on this subject in Ben-Bassat et al. (Journal of Bacteriology 169, 751-757 (1987)), O'Regan et al. (Gene 77, 237-251 (1989)), Sahin-Toth et al. (Protein Sciences 3, 240-247 (1994)) and Hochuli et al. (Bio/Technology 6, 1321-1325 (1988)), inter alia, and in well-known textbooks on genetics and molecular biology.

Amino acid sequences which correspondingly result from SEQ ID No. 2 also form part of the invention.

Likewise, DNA sequences which hybridize with SEQ ID No. 1 or portions of SEQ ID No. 1 form part of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers resulting from SEQ ID No. 1 form part of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Those skilled in the art will find instructions on the identification of DNA sequences by means of hybridization in inter alia the manual entitled "The DIG System User's Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41, 255-260), inter alia. Hybridization takes place under stringent conditions; in other words, only hybrids for which the probe and the target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

The hybridization reaction can be carried out for example using a 5x SSC buffer at a temperature of approx. 50°C - 68°C, it also being possible for probes to hybridize with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved for example by lowering the salt concentration to 2x SSC and subsequently to 0.5x SSC if necessary (The DIG System User's Guide for Filter Hybridization, Boehringer Mannheim, Mannheim, Germany,

1995), the temperature being adjusted to approx. 50°C - 68°C. It is possible to lower the salt concentration to 0.1x SSC if necessary. By raising the hybridization temperature in approx. 1 - 2°C steps from 50°C to 68°C, it is possible to isolate polynucleotide fragments which are e.g. at least 70%, at least 80% or at least 90% to 95% identical to the sequence of the probe used. Further instructions on hybridization are commercially available in the form of kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1603558).

Those skilled in the art will find instructions on the amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) in the manual by Gait entitled Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994), inter alia.

It has been found that, after overexpression of the pknD gene, the production of amino acids by corynebacteria is improved.

Overexpression can be achieved by increasing the copy number of the appropriate genes or mutating the promoter and regulatory region or the ribosome binding site located upstream from the structural gene. Expression cassettes incorporated upstream from the structural gene work in the same way. Inducible promoters additionally make it possible to increase the expression in the course of the production of amino acid by fermentation. Measures for prolonging the life of the mRNA also improve the expression. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme protein. The genes or gene constructs can either be located in plasmids of variable copy number or integrated and amplified in the chromosome. Alternatively, it is also possible to achieve overexpression of the genes in question

by changing the composition of the media and the culture technique.

Those skilled in the art will find relevant instructions in Martin et al. (Bio/Technology 5, 137-146 (1987)), Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), Eikmanns et al. (Gene 102, 93-98 (1991)), EP 0 472 869, US 4,601,893, Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), WO 96/15246, Malumbres et al. (Gene 134, 15-24 (1993)), JP-A-10-229891, Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) and Makrides (Microbiological Reviews 60, 512-538 (1996)), inter alia, and in well-known textbooks on genetics and molecular biology.

For amplification, the pknD gene according to the invention has been overexpressed for example with the aid of episomal plasmids. Suitable plasmids are those which are replicated in corynebacteria. Numerous known plasmid vectors, e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64, 549-554), pEKEx1 (Eikmanns et al., Gene 102, 93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107, 69-74 (1991)), are based on cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, e.g. those based on pCG4 (US-A-4,489,160), pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)) or pAG1 (US-A-5,158,891), can be used in the same way.

Other suitable plasmid vectors are those which make it possible to use the gene amplification process by integration into the chromosome, as described for example by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for the duplication or amplification of the hom-thrB operon. In this method the complete gene is cloned into a plasmid vector which can

replicate in a host (typically E. coli), but not in C. glutamicum. Examples of suitable vectors are pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994), Journal of Biological Chemistry 269, 32678-84; US-A-5,487,993), pCR®Blunt (Invitrogen, Groningen, The Netherlands; Bernard et al., Journal of Molecular Biology 234, 534-541 (1993)), pEM1 (Schrumpf et al., 1991, Journal of Bacteriology 173, 4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41, 337-342). The plasmid vector containing the gene to be amplified is then transferred to the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is described for example in Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods of transformation are described for example in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a crossover event, the resulting strain contains at least two copies of the gene in question.

It has also been found that amino acid exchanges in the section between position 661 and position 669 of the amino acid sequence of protein kinase D, shown in SEQ ID No. 2, improve the production of amino acids, especially lysine, by corynebacteria.

Preferably, L-glutamic acid in position 664 is exchanged for any other proteogenic amino acid except L-glutamic acid, and/or glycine in position 666 is exchanged for any other proteogenic amino acid except glycine.

The exchange in position 664 is preferably for L-lysine or L-arginine, especially L-lysine, and the exchange in

position 666 is preferably for L-serine or L-threonine, especially L-serine.

SEQ ID No. 3 shows the base sequence of the pknD-1547 allele contained in the strain DM1547. The pknD-1547 allele codes for a protein whose amino acid sequence is shown in SEQ ID No. 4. The protein contains L-lysine in position 664 and L-serine in position 666. The DNA sequence of the pknD-1547 allele (SEQ ID No. 3) contains the base adenine in place of the base guanine contained in the pknD wild-type gene (SEQ ID No. 1) in position 2501, and the base adenine in place of the base guanine in position 2507.

Mutagenesis can be carried out by conventional methods using mutagenic substances such as N-methyl-N'-nitro-N-nitrosoguanidine or ultraviolet light. Mutagenesis can also be carried out using in vitro methods such as treatment with hydroxylamine (Miller, J.H.: A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992) or mutagenic oligonucleotides (T.A. Brown: Gentechnologie für Einsteiger (Gene Technology for Beginners), Spektrum Akademischer Verlag, Heidelberg, 1993), or the polymerase chain reaction (PCR) as described in the manual by Newton and Graham (PCR, Spektrum Akademischer Verlag, Heidelberg, 1994).

The corresponding alleles or mutations are sequenced and introduced into the chromosome by the method of gene replacement, for example as described in Peters-Wendisch et al. (Microbiology 144, 915-927 (1998)) for the pyc gene of C. glutamicum, in Schäfer et al. (Gene 145, 69-73 (1994)) for the hom-thrB gene region of C. glutamicum or in Schäfer et al. (Journal of Bacteriology 176, 7309-7319 (1994)) for the cg1 gene region of C. glutamicum. The corresponding alleles or the associated proteins can optionally be amplified in turn.

In addition it can be advantageous for the production of L-amino acids to amplify and, in particular, overexpress not only the pknD gene but also one or more enzymes of the particular biosynthetic pathway, the glycolysis, the anaplerosis, the citric acid cycle, the pentose phosphate cycle or the amino acid export, and optionally regulatory proteins.

Thus, for the production of L-amino acids, one or more endogenous genes selected from the following group can be amplified and, in particular, overexpressed in addition to amplification of the pknD gene:

- the dapA gene coding for dihydrodipicolinate synthase (EP-B-0 197 335),
- the gap gene coding for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174, 6076-6086),
- the tpi gene coding for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174, 6076-6086),
- the pgk gene coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174, 6076-6086),
- the zwf gene coding for glucose-6-phosphate dehydrogenase (JP-A-09224661),
- the pyc gene coding for pyruvate carboxylase (DE-A-198 31 609),
- the lysC gene coding for a feedback-resistant aspartate kinase (Accession no. P26512; EP-B-0387527; EP-A-0699759),
- the lysE gene coding for lysine export (DE-A-195 48 222),

- The hom gene coding for homoserine dehydrogenase (EP-A-0131171),

the ilvA gene coding for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072)) or the ilvA(Fbr) allele coding for a feedback-resistant threonine dehydratase (Möckel et al., (1994), Molecular Microbiology 13, 833-842),

the ilvBN gene coding for acetohydroxy acid synthase (EP-B-0356739),

- the ilvD gene coding for dihydroxy acid dehydratase (Sahm and Eggeling (1999), Applied and Environmental Microbiology 65, 1973-1979),

- the zwa1 gene coding for the Zwa1 protein (DE 199 59 328.0, DSM13115).

In addition to amplification of the pknD gene, it can also be advantageous for the production of L-amino acids to attenuate one or more genes selected from the following group:

- the pck gene coding for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM13047),

- the pgi gene coding for glucose-6-phosphate isomerase (US 09/396,478, DSM12969),

- the poxB gene coding for pyruvate oxidase (DE 199 51 975.7, DSM13114),

- the zwa2 gene coding for the Zwa2 protein (DE 199 59 327.2, DSM13113),

and, in particular, to reduce the expression.

In this context the term "attenuation" describes the reduction or switching-off of the intracellular activity,

in a microorganism, of one or more enzymes (proteins) which are coded for by the appropriate DNA, for example by using a weak promoter or using a gene or allele coding for an appropriate enzyme with a low activity, or inactivating the appropriate gene or enzyme (protein), and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

It can also be advantageous for the production of amino acids not only to overexpress the pknD gene but also to switch off unwanted secondary reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention are also provided by the invention and can be cultivated for the production of amino acids continuously or discontinuously by the batch process, the fed batch process or the repeated fed batch process. A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Bioprocess Technology 1. Introduction to Bioengineering) (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Bioreactors and Peripheral Equipment) (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium to be used must appropriately meet the demands of the particular strains. Descriptions of culture media for various microorganisms can be found in "Manual of

Methods for General Bacteriology" of the American Society for Bacteriology (Washington DC, USA, 1981).

Carbon sources which can be used are sugars and carbohydrates, e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, e.g. soybean oil, sunflower oil, groundnut oil and coconut fat, fatty acids, e.g. palmitic acid, stearic acid and linoleic acid, alcohols, e.g. glycerol and ethanol, and organic acids, e.g. acetic acid. These substances can be used individually or as a mixture.

Nitrogen sources which can be used are organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used individually or as a mixture.

Phosphorus sources which can be used are phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogen-phosphate or the corresponding sodium salts. The culture medium must also contain metal salts, e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth-promoting substances such as amino acids and vitamins can be used in addition to the substances mentioned above. Suitable precursors can also be added to the culture medium. Said feed materials can be added to the culture all at once or fed in appropriately during cultivation.

The pH of the culture is controlled by the appropriate use of basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acidic compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled using antifoams such as fatty acid polyglycol esters. The stability of plasmids can be maintained by

adding suitable selectively acting substances, e.g. antibiotics, to the medium. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gaseous mixtures, e.g. air, into the culture. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. The culture is continued until the formation of the desired product has reached a maximum. This objective is normally achieved within 10 hours to 160 hours.

Methods of determining L-amino acids are known from the state of the art. They can be analyzed for example by ion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry 30 (1958) 1190), or by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51, 1167-1174).

A pure culture of the Corynebacterium glutamicum strain DM1547 was deposited as DSM 13994 in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures (DSMZ), Brunswick, Germany) on 16 January 2001 under the terms of the Budapest Treaty.

A pure culture of the Escherichia coli strain S17-1/pK18mobsacB_pknD_XL was deposited as DSM 14410 in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) on 18 July 2001 under the terms of the Budapest Treaty.

The fermentation process according to the invention is used for the preparation of amino acids.

The present invention is illustrated in greater detail below by means of Examples.

The isolation of plasmid DNA from Escherichia coli and all the techniques of restriction, Klenow treatment and alkaline phosphatase treatment were carried out according to Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA). Methods of transforming Escherichia coli are also described in this manual.

The composition of common nutrient media, such as LB or TY medium, can also be found in the manual by Sambrook et al.

Example 1

Preparation of a genomic cosmid gene library from Corynebacterium glutamicum ATCC13032

Chromosomal DNA from Corynebacterium glutamicum ATCC13032 was isolated as described by Tauch et al. (1995, Plasmid 33, 168-179) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, code no. 1758250). The DNA of cosmid vector SuperCos1 (Wahl et al. (1987), Proceedings of the National Academy of Sciences USA 84, 2160-2164), obtained from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vector Kit, code no. 251301), was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, product description XbaI, code no. 27-0948-02) and also dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, code no. 27-0868-04). The cosmid DNA treated in this way was mixed with the treated ATCC13032 DNA and the mixture was treated with T4 DNA ligase

(Amersham Pharmacia, Freiburg, Germany, product description T4 DNA ligase, code no. 27-0870-04). The ligation mixture was then packaged into phages using Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al., 1988, Nucleic Acid Research 16, 1563-1575), the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. Infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells being plated on LB agar (Lennox, 1955, Virology 1, 190) containing 100 mg/l of ampicillin. After incubation overnight at 37°C, recombinant single clones were selected.

Example 2

Isolation and sequencing of the pknD gene

The cosmid DNA of a single colony was isolated with the Qiaprep Spin Miniprep Kit (product no. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, product no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, product no. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range from 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (product no. 20021, Qiagen, Hilden, Germany).

The DNA of sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, product description Zero Background Cloning Kit, product no. K2500-01), was

cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, product no. 27-0868-04). Ligation of the cosmid fragments into sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then introduced into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences USA 87, 4645-4649) by electroporation (Tauch et al. 1994, FEMS Microbiol. Letters 123, 343-7) and plated on LB agar (Lennox, 1955, Virology 1, 190) containing 50 mg/l of zeocin.

Plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (product no. 900200, Qiagen, Hilden, Germany). Sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences USA 74, 5463-5467) with modifications by Zimmermann et al. (1990, Nucleic Acids Research 18, 1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (product no. 403044, Weiterstadt, Germany) was used. Separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphorese NF acrylamide/bisacrylamide" gel (29:1) (product no. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden programming package (1986, Nucleic Acids Research 14, 217-231), version 97-0. The individual sequences of the pZero-1 derivatives were assembled into a cohesive contig. Computer-assisted coding region analysis

was performed with the XNIP program (Staden, 1986, Nucleic Acids Research 14, 217-231).

The nucleotide sequence obtained is shown in SEQ ID No. 1. Analysis of the nucleotide sequence gave an open reading frame of 2223 base pairs, which was called the pknD gene. The pknD gene codes for a protein of 740 amino acids.

Example 3

Preparation of a replacement vector for replacement of the pknD allels

Chromosomal DNA was isolated from the strain DSM13994 by the method of Eikmanns et al. (Microbiology 140:1817-1828 (1994)). On the basis of the sequence of the pknD gene known for C. glutamicum from example 2, the following oligonucleotides were chosen for the polymerase chain reaction (see also SEQ ID No. 5 and SEQ ID No. 6):

pknD_XL-A1:
5` (tct aga) cgg ttg gtg gtt cgg ttc ag 3`
pknD_XL-E1:
5` (tct aga) agc ggc aat gcc ggt gag ta 3`

The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out by the PCR method of Karreman (BioTechniques 24:736-742, 1998) with Pwo-Polymerase from Boehringer. The primers pknD_XL-A1 and pknD_XL-E1 each contain an inserted cleavage site for the restriction enzyme XbaI, these being indicated in parentheses in the representation. With the aid of the polymerase chain reaction, a 1.6 kb DNA section is amplified and isolated, this carrying the pknD gene or allele.

The amplified DNA fragment of approx. 1.6 kb length, which carries the pknD allele of the strain DSM13994, was cleaved with the restriction enzyme XbaI, identified by

electrophoresis in a 0.8% agarose gel, isolated from the gel and purified by the conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The plasmid pK18mobsacB (Jäger et al., Journal of Bacteriology, 1:784-791 (1992)) was also cleaved with the restriction enzyme XbaI. The plasmid pK18mobsacB and the PCR fragment were ligated. The E. coli strain S17-1 (Simon et al., 1993, Bio/Technology 1:784-791) was then electroporated with the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. I, IRL-Press, Oxford, Washington DC, USA, 1985). Selection of plasmid-carrying cells was carried out by plating out the transformation batch on LB Agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme XbaI and subsequent agarose gel electrophoresis (0.8%). The plasmid was called pK18mobsacB_pknD_XL and is shown in Figure 1.

Brief Description of the Figure:

Figure 1: Map of the plasmid pK18mobsacB_pknD_XL.

The abbreviations and designations used have the following meaning. The length data are to be understood as approx. values.

sacB:	sacB gene
oriV:	Replication origin V
KmR:	Kanamycin resistance
XbaI:	Cleavage site of the restriction enzyme XbaI

pknD´: Incomplete fragment of the pknD gene from DM1547

What is claimed is:

1. An isolated polynucleotide from corynebacteria which contains a polynucleotide sequence coding for the pknD gene and is selected from the group comprising:

a) a polynucleotide which is at least 70% identical to a polynucleotide coding for a polypeptide containing the amino acid sequence of SEQ ID No. 2,

b) a polynucleotide coding for a polypeptide containing an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2,

c) a polynucleotide which is complementary to the polynucleotides of a) or b), and

d) a polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of protein kinase D.

2. A polynucleotide as claimed in claim 1 which is a preferably recombinant DNA replicable in corynebacteria.

3. A polynucleotide as claimed in claim 1 which is an RNA.

4. A polynucleotide as claimed in claim 2 which contains the nucleic acid sequence as shown in SEQ ID No. 1.

5. A replicatable DNA as claimed in claim 2 which contains:

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence corresponding to sequence (i) within the degeneracy of the genetic code, or

(iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

(iv) neutral sense mutations in (i).

6. A replicable DNA as claimed in claim 5 wherein the hybridization is carried out under a stringency corresponding to at most 2x SSC.

7. A polynucleotide sequence as claimed in claim 1 which codes for a polypeptide containing the amino acid sequence shown in SEQ ID No. 2.

8. Corynebacteria in which the pknD gene is amplified and, in particular, overexpressed.

9. A fermentation process for the preparation of L-amino acids, especially L-lysine, wherein the following steps are carried out:

a) fermentation of the corynebacteria producing the desired L-amino acid, in which at least the endogenous pknD gene or nucleotide sequences coding therefor are amplified and, in particular, overexpressed,

b) enrichment of the L-amino acid in the medium or in the cells of the bacteria, and

c) isolation of the L-amino acid.

10. The process as claimed in claim 9 wherein bacteria are used in which other genes of the biosynthetic pathway of the desired L-amino acid are additionally amplified.

11. The process as claimed in claim 9 wherein bacteria are used in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partially switched off.

12. The process as claimed in claim 9 wherein a strain transformed with a plasmid vector is used and the plasmid vector carries the nucleotide sequence coding for the pknD gene.

13. The process as claimed in claim 9 wherein the expression of the polynucleotide(s) coding for the pknD gene is amplified and, in particular, overexpressed.

14. The process as claimed in claim 9 wherein the catalytic properties of the polypeptide (enzyme protein) for which the pknD polynucleotide codes are enhanced.

15. The process as claimed in claim 9 wherein, for the production of L-amino acids, coryneform microorganisms are fermented in which one or more endogenous genes selected from the following group are simultaneously amplified and, in particular, overexpressed:

15.1 the dapA gene coding for dihydrodipicolinate synthase,

15.2 the gap gene coding for glyceraldehyde 3-phosphate dehydrogenase,

15.3 the tpi gene coding for triose phosphate isomerase,

15.4 the pgk gene coding for 3-phosphoglycerate kinase,

15.5 the zwf gene coding for glucose-6-phosphate dehydrogenase,

15.6 the pyc gene coding for pyruvate carboxylase,

15.7 the lysC gene coding for a feedback-resistant aspartate kinase,

15.8 the lysE gene coding for lysine export,

15.9 the hom gene coding for homoserine dehydrogenase,

15.10 the ilvA gene coding for threonine dehydratase or the ilvA(Fbr) allele coding for a feedback-resistant threonine dehydratase,

15.11 the ilvBN gene coding for acetohydroxy acid synthase,

15.12 the ilvD gene coding for dihydroxy acid dehydratase, or

15.13 the zwa1 gene coding for the Zwa1 protein.

16. The process as claimed in claim 9 wherein, for the production of L-amino acids, coryneform microorganisms are fermented in which one or more genes selected from the following group are simultaneously attenuated:

16.1 the pck gene coding for phosphoenol pyruvate carboxykinase,

16.2 the pgi gene coding for glucose-6-phosphate isomerase,

16.3 the poxB gene coding for pyruvate oxidase, or

16.4 the zwa2 gene coding for the Zwa2 protein.

17. Escherichia coli strain S17-1/pK18mobsacB_pknD_XL as DSM 14410 deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Brunswick, Germany.

18. Corynebacteria which contain a vector carrying a polynucleotide as claimed in claim 1.

19. The process as claimed in one or more of claims 9-16, wherein microorganisms of the species Corynebacterium glutamicum are used.

20. The process as claimed in claim 19, wherein the Corynebacterium strain S17-1/pK18mobsacB_pknD_XL is used.

21. A method of detecting RNA, cDNA and DNA in order to isolate nucleic acids, or polynucleotides or genes, which code for protein kinase D or have a high degree of similarity to the sequence of the pknD gene, wherein the polynucleotide containing the polynucleotide sequences as claimed in claims 1, 2, 3 or 4 is used as hybridization probes.

22. The method as claimed in claim 21 wherein arrays, micro-arrays or DNA chips are used.

23. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences between positions 661 and 669 in SEQ ID No. 2 are modified by amino acid exchange.

24. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences contain any other proteogenic amino acid except glutamic acid in position 664 in SEQ ID No. 2.

25. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences contain L-lysine or L-arginine in position 664 in SEQ ID No. 2.

26. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid

sequence contains L-lysine in position 664 in SEQ ID No. 2.

27. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences contain any other proteogenic amino acid except glycine in position 666 in SEQ ID No. 2.

28. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequences contain L-serine or L-threonine in position 666 in SEQ ID No. 2.

29. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequence contains L-serine in position 666 in SEQ ID No. 2.

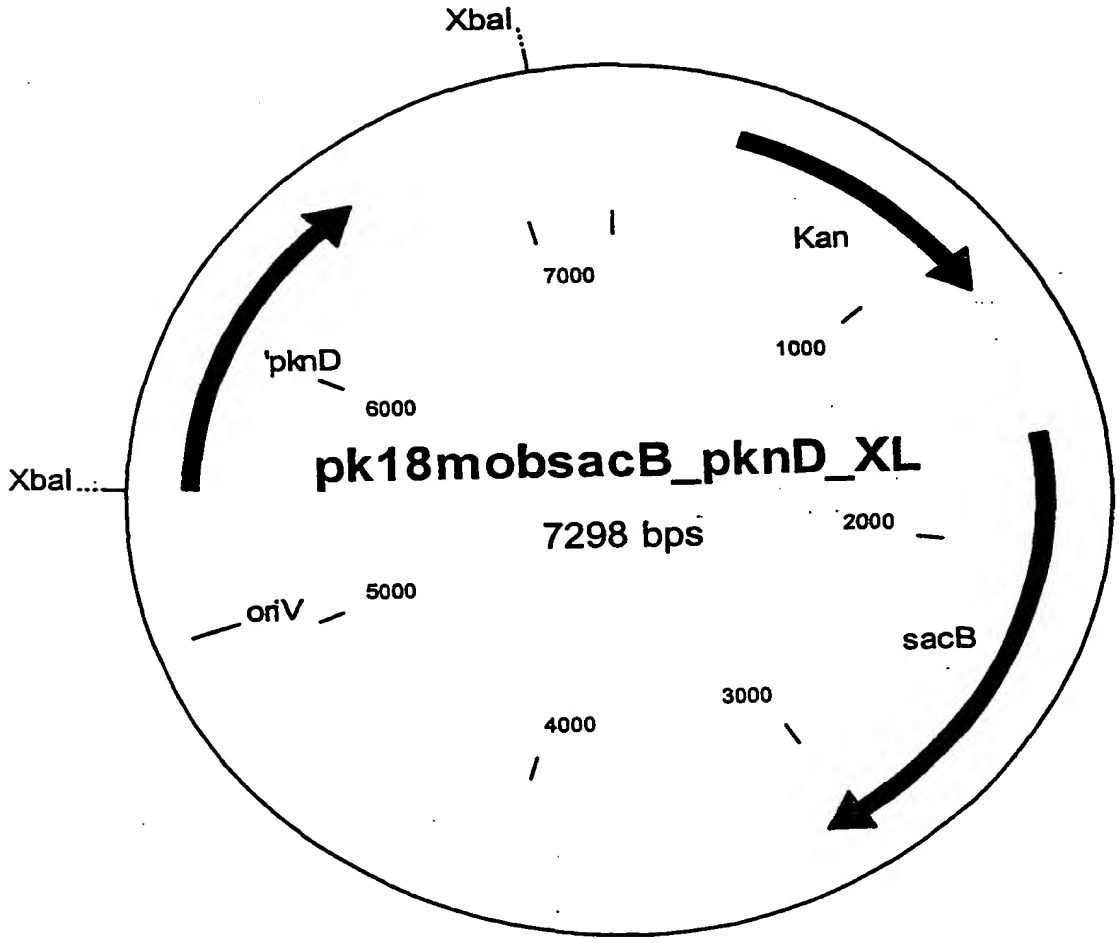
30. A DNA originating from corynebacteria and coding for protein kinase D, wherein the corresponding amino acid sequence contains glycine in position 664 and L-serine in position 666, shown in SEQ ID No. 4.

31. A DNA as claimed in claim 30 wherein said DNA contains the nucleobase adenine in position 2501 and the nucleobase adenine in position 2507, shown in SEQ ID No. 3.

32. Corynebacteria which contain a DNA as claimed in one or more of claims 23 to 31.

33. Corynebacterium glutamicum DM1547 deposited as DSM13994 in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Brunswick, Germany.

Figure 1: Map of the plasmid pk18mobsacB pknD XL



SEQUENCE LISTING

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cgctgaagcc accgtcggca agcaaggcaa taactccagg gatgaaacgg ttggtgtttt 180

ccttcttggt gctcaggaaa gcttctggaa tgtagcgaat accgtcgcgc cggaccacga 240

tcaatttgtg ttcattgacc agatccatca ccttggtgac aacaacgccg aggcgctcgg 300

ctgtctccgg aagggtcagc aatggttcat tgtcgggcag ggcgaaggaa gattcattgt 360

tggaactcac agtcttaatt tagctggttc gagctctaat ggagaatctt tagggtattt 420

ctgcgcgtgc cgggaatgaa agcaccttct tgacctttga aaacaggatg tcactaccac 480

tttttgtgta ccttccgaca tactggaacg c atg gca aac ttg aag gtc ggt 532
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1 5

gac gtt tta gag gac agg tat cgg att gaa act ccg att gcc cgg ggt 580
Asp Val Leu Glu Asp Arg Tyr Arg Ile Glu Thr Pro Ile Ala Arg Gly
10 15 20

ggt atg tct acc gtg tac agg tgc ctt gat ctt cgt tta gga cgt tcc 628
Gly Met Ser Thr Val Tyr Arg Cys Leu Asp Leu Arg Leu Gly Arg Ser
25 30 35

atg gcg ctt aaa gtc atg gaa gaa gat ttc gtt gat gat ccc att ttc 676
Met Ala Leu Lys Val Met Glu Glu Asp Phe Val Asp Asp Pro Ile Phe
40 45 50 55

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cca aat ttg gtc aat gtg tat gat ttt tcc gct act gac ggt ttg gtg   772
Pro Asn Leu Val Asn Val Tyr Asp Phe Ser Ala Thr Asp Gly Leu Val
            75                  80                  85

tat ctg gtg atg gag tta atc act ggt ggc acc ttg cgt gag ttg ctg   820
Tyr Leu Val Met Glu Leu Ile Thr Gly Gly Thr Leu Arg Glu Leu Leu
        90                  95                  100

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ggg gtg ctc acg ggt ctc gcg gct gcc cac cgg gcg ggc atg gtg cac   916
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120                 125                 130                 135

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            235                 240                 245

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ccc aag act cct gag cct gat cac act gcg atc att ccg gtg gcc tca   1492
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Ala Asn Glu Thr Ser Ile Leu Pro Ala Gln Asn Met Ala Gln Asn Met
    330                 335                 340

gcg cag aat ccg ctg caa cct ccg gaa cct gat ttc gcc ccg gag cca   1588
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345                 350                 355

cct ccg gac aca gcg ctg aat att caa gat caa gag ctt gcg cgc gcc   1636
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360                 365                 370                 375

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            380                 385                 390

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Thr Leu Trp Ser Ile Phe Val Val Ala Val Ile Ala Ala Val Ala Val
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gcg gac gac aac gat cga att tat agt caa acc cct cgt gca cgc agc 2692
Ala Asp Asp Asn Asp Arg Ile Tyr Ser Gln Thr Pro Arg Ala Arg Ser
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gaa gtc tcg gta ggg gga gaa gtt aca gta agg gcg ttt tagtggttcc 2741
Glu Val Ser Val Gly Gly Glu Val Thr Val Arg Ala Phe
730 735 740

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ccttcttggt gctcaggaaa gcttctggaa tgtagcgaat accgtcgcgc cggaccacga 240

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ctgtctccgg aagggtcagc aatggttcat tgtcgggcag ggcgaaggaa gattcattgt 360

tggaactcac agtcttaatt tagctggttc gagctctaat ggagaatctt tagggtattt 420

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Gly Met Ser Thr Val Tyr Arg Cys Leu Asp Leu Arg Leu Gly Arg Ser
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atg gcg ctt aaa gtc atg gaa gaa gat ttc gtt gat gat ccc att ttc 676
Met Ala Leu Lys Val Met Glu Glu Asp Phe Val Asp Asp Pro Ile Phe
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cgg cag cgt ttc cgt agg gaa gct cgg tca atg gcg cag cta aat cat 724
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Tyr Leu Val Met Glu Leu Ile Thr Gly Gly Thr Leu Arg Glu Leu Leu
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gct gag cgg gga cct atg ccc ccg cat gct gct gtg ggc gtt atg cgt 868
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gat gat ctc gac cat gca tac gcc cgc ctt acg gaa gtc gtg ccg gca   1204
Asp Asp Leu Asp His Ala Tyr Ala Arg Leu Thr Glu Val Val Pro Ala
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ccg agt tcg ctt atc gac ggc gtc ccc tcc ctc atc gat gag ctt gtc   1252
Pro Ser Ser Leu Ile Asp Gly Val Pro Ser Leu Ile Asp Glu Leu Val
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Pro Lys Thr Pro Glu Pro Asp His Thr Ala Ile Ile Pro Val Ala Ser
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gcg cag aat ccg ctg caa cct ccg gaa cct gat ttc gcc ccg gag cca   1588
Ala Gln Asn Pro Leu Gln Pro Pro Glu Pro Asp Phe Ala Pro Glu Pro
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cct ccg gac aca gcg ctg aat att caa gat caa gag ctt gcg cgc gcc   1636
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acg ttg tgg tca att ttc gtg gtc gca gtg atc gct gct gtt gct gtt   1732
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Gly Leu Thr Leu Thr Thr Asp Ala Asp Asp Asn Asp Arg Ile Tyr Ser
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number
WO 02/22632 A3

(51) International Patent Classification[7]: C12N 15/31, C12P 13/04, 13/08, C12Q 1/68 // C12R 1:15

(21) International Application Number: PCT/EP01/10210

(22) International Filing Date: 5 September 2001 (05.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
100 44 948.4 12 September 2000 (12.09.2000) DE
101 20 094.3 25 April 2001 (25.04.2001) DE

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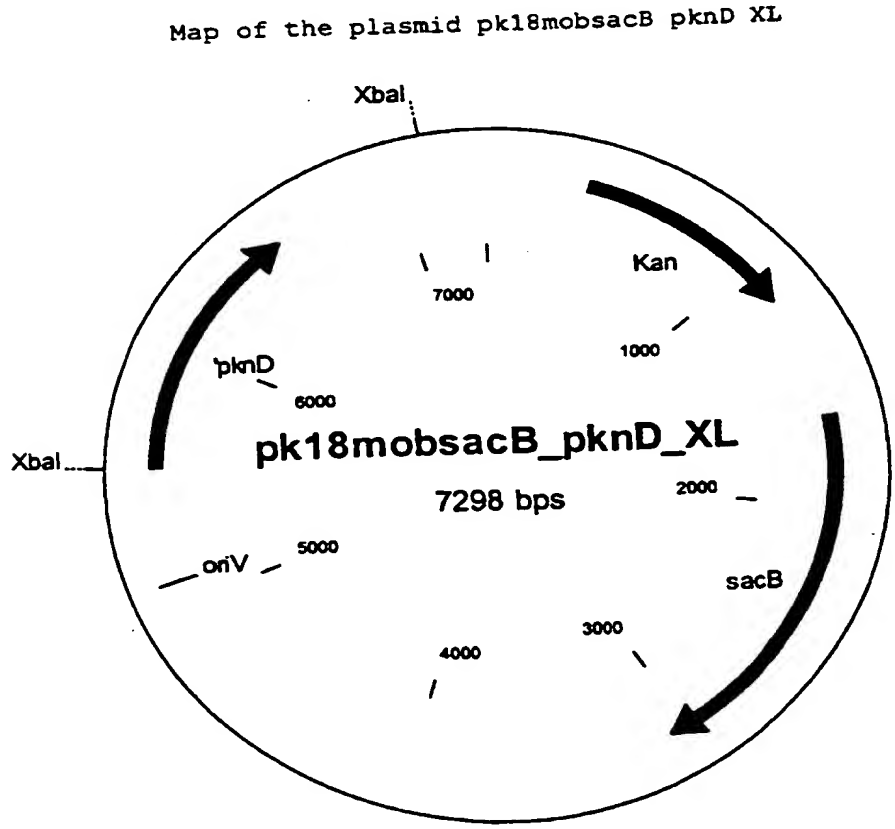
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,

[Continued on next page]

(54) Title: NUCLEOTIDE SEQUENCES CODING FOR THE PKND GENE



(57) Abstract: The invention relates to an isolated polynucleotide which contains a polynucleotide sequence selected from the group comprising: a) a polynucleotide which is at least 70% identical to a polynucleotide coding for a polypeptide containing the amino acid sequence of SEQ ID No. 2, b) a polynucleotide coding for a polypeptide containing an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2, c) a polynucleotide which is complementary t the polynucleotides of a) or b), and d) a polynucleotide containing at least 15 consecutive nucleotides of the polynucleotide sequence of a), b) or c), and a fermentati n process for the preparation of L-amino acids using corynebacteria in which at least the pknD gene is amplified, and to the use, as hybridization probes, of polynucleotides containing the sequences according to the invention.

WO 02/22632 A3

IT, LU, MC, NL, PT, SE, TR). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the internati nal search report:
13 June 2002

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/10210

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12P13/04 C12P13/08 C12Q1/68 //C12R1:15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12R C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, SEQUENCE SEARCH, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 411 997 A (SHIMAZAKI KEISHI ET AL) 25 October 1983 (1983-10-25) the whole document ---	8-11, 13-16
X	WO 00 17379 A (CHEIL JEDANG CORP ;LEE JAE HEUNG (KR); LIM SANG JO (KR); KO JUNG H) 30 March 2000 (2000-03-30) the whole document ---	8-11, 13-16
X	DATABASE EMBL 'Online! MTV021 Accession Number AL021957, 23 February 1998 (1998-02-23) COLE ET AL. : "Mycobacterium tuberculosis H37Rv complete genome; segment 97/162" XP002191212 accession number AL021957 --- -/--	1-4

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
8 April 2002	22/04/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Kools, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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P,X	DATABASE EMBL 'Online! Accession number AX125968, 10 May 2001 (2001-05-10) NAKAGAWA ET AL.: "Sequence 5884 from Patent EP1108790" XP002191701 accession number AX125968	1-33
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INTERNATIONAL SEARCH REPORT

information on patent family members

Inte ional Application No
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